INFECTION AND IMMUNITY, Sept. 1987, p. 1963–1969
0019-9567/87/091963-07$02.00/0

Vol. 55, No. 9

Evaluation with an *iuc*::Tn*10* Mutant of the Role of Aerobactin Production in the Virulence of *Shigella flexneri*

XAVIER NASSIF,[1] MARIE-CHRISTINE MAZERT,[2] JOËLLE MOUNIER,[1] AND PHILIPPE J. SANSONETTI[1]*

Service des Entérobactéries, Institut National de la Santé et de la Recherche Médicale U199, Institut Pasteur, 75724 Paris Cedex 15,[1] and Pasteur Vaccins, 92430 Marnes-La-Coquette,[2] France

Received 9 February 1987/Accepted 16 May 1987

To evaluate the role of aerobactin production in the virulence of *Shigella flexneri*, a *iuc*::Tn*10* insertion mutant was obtained from strain M90T, a serotype 5 isolate. This mutant was tested for its ability to invade and kill HeLa cells in monolayers, to elicit keratoconjunctivitis in guinea pigs, and to infect ligated segments of rabbit ileal loops. Although this mutant did not grow in iron-depleted media, its ability to grow intracellulalry and eventually kill HeLa cells was unchanged from that of the wild-type strain. On the other hand, an inoculum-dependent effect was observed in the Sereny test, as well as in the rabbit ligated ileal loop model, which was monitored for fluid production and for both gross and microscopical alterations of the mucosa. Transduction of the mutation within a noninvasive plasmidless derivative of the parental strain did not alter growth within the intestinal lumen. We conclude that aerobactin production most probably provides invasive strains with a selective advantage for growth within tissues when located in extracellular compartments.

The pathogenesis of bacillary dysentery comprises several critical stages. In the first stage shigellae must have a selective advantage over the indigenous flora and an ability to reach and survive on the mucosal surface. The second stage involves the invasion of colonic epithelial cells and encompasses entry into cells, intracellular multiplication, and eventual cell killing. This leads to the final stage, that of dissemination within the conjunctive tissues of the lamina propria, in which bacterial invasion triggers a strong inflammatory reaction which causes superficial abscesses and ulcerations. Molecular products that allow bacterial survival and growth on the surface of the colonic mucosa, within epithelial cells, and finally within conjunctive tissues are largely unknown. In *Shigella flexneri*, a 220-kilobase plasmid encodes functions for bacterial entry into cells and subsequent lysis, phenomena correlated with the ability of intracellular bacteria to replicate freely within the cytoplasm (24, 25). More recent studies on the intracellular behavior of *S. flexneri* in macrophages have suggested that early killing of host cells is also correlated with rapid lysis of the phagocytic vacuole, thus allowing direct expression of other virulence genes within the cytosol (7). These data are not sufficient to answer all pending questions.

Previous experiments have shown that a segment adjacent to *mtl* on the chromosome of *S. flexneri* 2a is necessary for completing the invasive phenotype of an *Escherichia coli* K-12–*S. flexneri* 2a hybrid (23). Subsequent work associated genes encoding the hydroxamate siderophore aerobactin and its receptor with this region (9). Therefore it appeared reasonable to investigate the possibility that aerobactin is a critical virulence factor of *S. flexneri* with a potential role in bacterial survival within the intestinal lumen and within the intestinal tissues in which iron is chelated by transferrin. In addition, an iron chelator may also be critical for bacterial replication within cells in which iron is immobilized by ferritin, as well as for early cell killing through depletion of iron, which acts as a cofactor of enzymes critical for eucaryotic cell respiration. Such a mechanism has already been described for the process by which activated macrophages may kill tumor cells (10).

In a recent work, Lawlor et al. constructed a mutant defective in aerobactin production (14). Such a strain did not demonstrate alteration in its capacity to invade HeLa cells and multiply intracellularly. On the other hand, it seemed to be impaired in its capacity to grow in the extracellular compartment of embryonated eggs. In an attempt to define the stage of the infectious process at which the production of aerobactin could provide a selective advantage, we constructed a Tn*10* mutant of *S. flexneri* serotype 5 which no longer produced aerobactin (i.e., *iuc*::Tn*10*) and tested its virulence in a HeLa cell model and in rabbit ligated ileal loops. This mutant showed no significant alteration in its capacity to multiply intracellularly, as previously described, or in its ability to kill host cells. On the other hand, the mutation appeared to alter the growth capacity of the strain within tissues in the extracellular compartment, in an inoculum-dependent manner.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *S. flexneri* serotype 5 strains used in this study were M90T, which contains the virulence plasmid pWR100 (24), and its noninvasive derivative BS176, which has been cured of pWR100. *E. coli* LG1522 (*ara fepA lac leu mtl trpE proC rpsL supE thi tonA xyl*; pColV-K30*iuc*) (6) was used for aerobactin detection. The plasmids used were pABN1, which carries the aerobactin biosynthesis and transport genes of pColV-K30 (3), and F'(Ts)114*lac*::Tn*10*, which is a temperature-sensitive replicon carrying the tetracycline resistance transposon Tn*10* and the lactose operon (13). These plasmids were maintained in *E. coli* HB101 (5). Bacteria were routinely grown in L broth (16) or Trypticase soy broth (Diagnostics Pasteur, Marnes-La-Coquette, France). More specific experiments required MacConkey lactose agar (Difco Laboratories, Detroit, Mich.), Hektoen agar (Diagnostics Pasteur), or M9 medium (21) supplemented with glucose (2 g/liter), Casamino Acids (5 mg/ml; Difco), thiamine (50 μg/ml), and nicotinic acid (10 μg/ml). For experiments involving bacteriophage P1 *vir*, L

* Corresponding author.

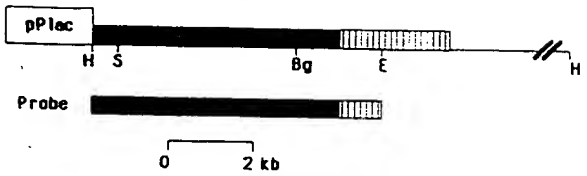

FIG. 1. Structure of pABN1. This is a recombinant plasmid carrying the aerobactin operon of the *E. coli* plasmid pColV-K30. The probe used contained the aerobactin biosynthesis genes (*iuc*) and a part of the ferric aerobactin receptor gene (*iut*). Symbols: ▬, sequences encoding aerobactin biosynthesis; ▭, sequences encoding the ferric-aerobactin receptor. —

broth, L agar, and L soft agar (0.65% agarose) were supplemented with 5 mM $CaCl_2$. Iron-limiting conditions were obtained by supplementing M9 medium with either 150 μM α,α′-dipyridyl or 150 μg of human transferrin per ml (both from Sigma Chemical Co., St. Louis, Mo.). When necessary, antibiotics were added at the following concentrations: tetracycline, 12.5 μg/ml; ampicillin, 100 μg/ml.

To test the ability of the strains to grow in deferrated medium, we made a preculture in M9 broth without deferration. A portion was then inoculated in deferrated M9 broth at a density of 10^7 cells per ml.

Assays for siderophore production. Enterochelin was detected by the technique of Arnow (1). Aerobactin production was detected by a biological assay with *E. coli* LG1522 as the indicator strain. This mutant is deficient in enterochelin uptake and aerobactin synthesis. It expresses the receptor for the ferric-aerobactin complex. Lawns of this culture (10^7 bacteria per plate) were spread onto supplemented M9 minimal agar plates containing α,α′-dipyridyl. Strains were then spotted onto the lawn. Aerobactin production was detected as a halo of satellite growth after overnight incubation at 37°C (6).

Isolation and cleavage of DNA. Plasmid DNA preparations of *S. flexneri* were made by the technique of Kado and Liu (12). The technique of Birnboim and Doly was used for plasmid DNA extraction in *E. coli* K-12 (4). Preparation of total bacterial DNA has been described previously (22). Restriction endonuclease digestion of DNA was performed as recommended by the manufacturers. Plasmid DNA and the restriction products were subjected to agarose gel electrophoresis (0.7%) in E buffer (40 mM Tris, 2 mM disodium EDTA).

Preparation of DNA probe and hybridization. A restriction fragment of pABN1, a recombinant plasmid which contains the aerobactin operon of plasmid pColV-K30, was used as probe (Fig. 1). The probe was purified by electrophoresis in low-melting-point agarose gel (0.7 to 1%). The DNA fragment was then labeled with ^{32}P (The Radiochemical Centre, Amersham, England) by nick translation (20). DNA to be hybridized was blotted from agarose gels onto nitrocellulose filters (Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany). Hybridizations were carried out by the method of Southern (27).

Conjugative transfer of plasmids. Conjugation was carried out at 37°C in static liquid cultures. Donor and recipient cells were mixed in a ratio of 1:10. Bacteria were allowed to conjugate for 2 to 4 h. Transconjugants were selected by using amino acid auxotrophs on M9 minimal medium.

Transduction methods. The P1 *vir* transducing lysate was prepared by the confluent plate technique (8). Recipient strains were infected with P1 at a multiplicity of 1 to 3; after adsorption for 20 min at 37°C the mixture was plated directly onto selective medium.

Isolation of an *icu* mutant of M90T. A *icu* mutant which no longer produced aerobactin was constructed by transposition of Tn*10* into the aerobactin biosynthesis sequence. F′(Ts)114*lac*::Tn*10* was used as the Tn*10* donor. It was conjugatively transferred at 32°C into the virulent *S. flexneri* M90T. A purified transconjugant, M90T(*F*′(Ts)114*lac*::Tn*10*), was cultured at 42°C on MacConkey lactose agar containing tetracycline. Lactose-negative, tetracycline-resistant clones, resulting from loss of the F′(Ts)114 plasmid vector and transposition of Tn*10*, were selected. A bank of mutants were then established and subsequently screened for *iuc* mutant clones. The M90T derivative which no longer produced aerobactin was named M90T *iuc*::Tn*10*. The mutation was then transduced with a P1 *vir* lysate into the noninvasive strain BS176. The *iuc* mutant of BS176 was named BS176*iuc*::Tn*10*.

Assay for intracellular growth of bacteria. Semiconfluent monolayers of HeLa cells were infected at a multiplicity of infection of about 50 bacteria per cell in 35-mm plastic tissue culture dishes (Becton Dickinson Labware, Oxnard, Calif.). The intracellular growth of bacteria was monitored as previously described (25), and 20 μg of gentamicin per ml was added to the medium to prevent any further reinfection of the cells. The number of bacteria per infected cell was obtained by using the following formula: (number of bacteria per plate)/(number of HeLa cells multiplied by the percentage of infected cells). For each strain the experiments were repeated twice and the mean values were calculated.

Assay for cellular killing. The assay for cellular killing was done as previously described (7) with modifications. Radiolabeled HeLa cells were used instead of macrophages. Before being infected they were labeled for 18 h in a culture medium containing 1 μCi of [^3H]uridine (The Radiochemical Centre) per ml; they were then infected as described above and incubated for 6 h in a medium containing 20 μg of gentamicin per ml. At that time cells were washed and lysed with 1 ml of 0.5% sodium deoxycholate in distilled water. A 100-μl portion of this lysate was counted in an NCS/ACS preparation (1:9 ratio) (The Radiochemical Centre). For each experiment a control dish containing uninfected cells was included. The percentage of residual HeLa cells on the dish was then determined by measuring the amount of radioactivity remaining in the infected dish compared with that remaining in the uninfected one. For each strain the experiments were repeated nine times and the mean values and standard deviations were calculated.

Sereny test. The ability of invasive bacteria to elicit keratoconjunctivitis in guinea pigs was tested as previously described (26). Inocula were carefully standardized from 10^5 to 10^8 bacteria. Identical inocula of the two tested strains (i.e., M90T and the *iuc* mutant) were injected into each eye of the same animal.

Rabbit ileal loop test. Rabbit ligated ileal loops of approximately 10 cm with 2-cm spacers were prepared in 1.76- to 2.04-kg rabbits which were anesthesized with a 6% solution of sodium pentobarbital (0.5 ml/kg). For each strain (i.e., M90T, M90T*iuc*::Tn*10*, and BS176), inocula of 10^7 and 10^9 CFU in 1 ml of Trypticase soy broth were tested. Each inoculum of each strain was tested in a minimum of four rabbits. Rabbits were sacrificed 18 h later. Loops were examined, fluid accumulation was recorded, and the volume-to-length ratio was calculated. After dissection, the condition of the mucosal surface was observed. Portions of the loops infected were fixed in 10% buffered Formalin. Speci-

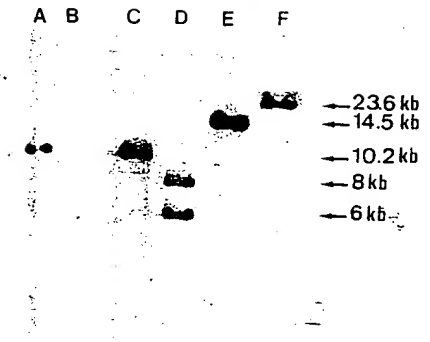

FIG. 2. Hybridization of *Shigella* DNA with the probe described in Fig. 1. Lanes: A, total DNA of M90T digested with *Hin*dIII; B, plasmid extract of M90T; C and D, total DNA of M90T and M90T*iuc*::Tn*10*, respectively, digested with *Hin*dIII; E and F, total DNA of M90T and M90T*iuc*::Tn*10*, respectively, digested with *Sal*I.

mens were processed by standard procedures and stained with hematoxylin-eosin-safranin. Observation of the preparation by microscopy was independently performed by two pathologists who were not aware of the infecting strain. With BS176 and BS176*iuc*::Tn*10*, which cannot invade the intestinal mucosa, the number of bacteria contained within loops after a 24-h growth was measured. For each of these two strains, two inocula were tested (10^7 and 10^9 CFU). The inoculum injected was prepared as described above. After the rabbits had been sacrificed, 10 ml of saline was introduced into each loop to recover intraluminal shigellae. The bacterial number within each loop was calculated after they had been plated on Hektoen agar, which allows easy recognition of *Shigella* colonies. Each inoculum of the two strains was tested in six different loops, and the mean values and standard deviations were calculated.

RESULTS

Characterization of the iron transport system in M90T. As was previously shown for other *S. flexneri* isolates (15, 18), M90T did not produce a phenolate siderophore as detected in the test described by Arnow (1). It produced a siderophore of the hydroxamate type that the bioassay identified as aerobactin. Hybridization of an M90T plasmid extract with the probe which contains the *iuc* genes encoding aerobactin (Fig. 1) did not localize these genes on pWR100 (Fig. 2, lane B). With the same experiment was performed on total DNA digested with *Hin*dIII (Fig. 2, lane A), a signal was obtained, indicating that the *iuc* genes were chromosomal.

Construction and characterization of the mutant M90T*iuc*::Tn*10*. M90T clones which had received Tn*10* but had lost the F'(Ts)114*lac* plasmid vector were selected and tested for aerobactin production. Of 394 mutants tested, 1 was found to be defective in hydroxamate production. This mutant, like the wild-type strain, grew well in M9 broth supplemented as described above, but did not grow in 48 h when a subculture was performed in the same medium in which iron was chelated with α,α'-dipyridyl or transferrin. To determine whether the ferric-aerobactin receptor was still expressed in the mutant, this strain was incorporated in an M9 minimal agar plate with the supplements described in Materials and Methods and with 150 μM α,α'-dipyridyl. The wild-type strain was then spotted onto the agar surface. After 24 h a zone of satellite growth appeared around M90T, showing that exogenous aerobactin could cross-feed the defective strain and that the ferric-aerobactin receptor was still expressed by the mutant. It was not possible to assess the presence of the ferric-aerobactin receptor by testing the sensitivity of the mutant to cloacin, because this strain cannot grow in the iron-depleted medium which is necessary for induction of the ferric-aerobactin receptor.

To demonstrate that the Tn*10* insertion was localized within the sequence encoding aerobactin biosynthesis, total DNA samples from the wild-type isolate and the *iuc* mutant were digested with *Hin*dIII, which cleaves Tn*10*, and with *Sal*I, which has no cleaving site within the transposon (11). Hybridization of such material was performed with the probe shown in Fig. 1. M90T digested with either of the two enzymes showed a single band of hybridization (Fig. 2, lanes C and E). On the other hand, mutant DNA showed two hybridization signals corresponding to smaller fragments when digested with *Hin*dIII (lane D) and one signal corresponding to a larger fragment when digested with *Sal*I (lane F). These results indicate that Tn*10* is localized within the *iuc* genes, thus increasing the size of one *Sal*I-digested fragment by approximately the transposon length and creating new *Hin*dIII restriction sites within one of the genes.

Effect of the *iuc* mutation on the bacterial capacity to grow intracellularly and kill cells. To evaluate the role of aerobactin in the ability of *S. flexneri* to grow intracellularly, we studied the respective growth kinetics of M90T and mutant M90T*iuc*::Tn*10* within HeLa cells. The results are reported in Fig. 3. No significant difference in growth between the wild-type strain and the mutant could be observed, indicating that aerobactin is not a crucial factor for intracellular growth.

In addition, the capacity of the two strains to promote early killing of host cells was monitored as a function of detachment of radiolabeled HeLa cells within 6 h after infection. The percentage of residual radioactivity on tissue culture dishes was 62 ± 8% with the wild-type strain and 61 ± 5% with the mutant. This indicated that the *iuc* mutation does not affect the capacity of early killing of host cells by *S. flexneri*.

Therefore on the basis of the HeLa cell model, aerobactin does not play a significant role in the intracellular behavior of *S. flexneri*.

In vivo virulence assays. To compare the in vivo virulence of the two strains, we used two assays: the Sereny test and infection of ligated segments of rabbit ileal loops.

The Sereny test was performed with different inocula of the two bacteria. Each guinea pig received an equivalent dose of the wild-type strain and the *iuc* mutant in each eye. The results are presented in Table 1. At the usual inoculum of 10^8 CFU, no significant difference could be observed between the two strains in their ability to elicit keratoconjunctivitis. At 10^7 CFU, M90T and the *iuc* mutant produced a normal response in 2 and 3 days, respectively. At 10^6 CFU the wild-type strain still elicited severe keratoconjunctivitis, while no response could be detected with the *iuc* mutant. These results indicate that on the basis of the Sereny test, the *iuc* mutation does not produce any qualitative alteration in the virulence of *S. flexneri*: only a quantitative modification could be observed, indicating that aerobactin production provides strains with a selective advantage over strains that do not produce this siderophore.

To further test the role of aerobactin production in the virulence of *S. flexneri* and attempt to define the stage of the infectious process at which aerobactin operates, we used a more definitive assay. Segments of rabbit ligated ileal loops

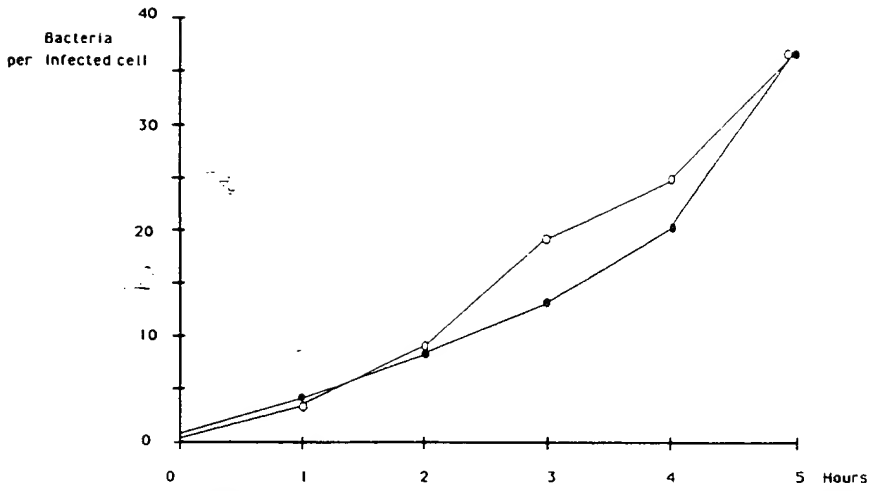

FIG. 3. Kinetics of bacterial growth of M90T and M90T*iuc*::Tn*10*. Each point is the mean of two different experiments. Symbols: ●, M90T; ○, M90T*iuc*::Tn*10*.

were infected with two inocula of each of the strains tested (10^7 and 10^9 CFU). Results were evaluated after overnight infection by measurement of fluid production (which is null in an uninfected loop), by gross examination of the mucosal surface of the opened loop, and by histopathological observation. The intraluminal fluid production is shown in Fig. 4. M90T produced similar amount of fluid with the two inocula tested. At an inoculum of 10^9 CFU the *iuc* mutant elicited a lower response, although the difference was not statistically significant. However, a striking decrease could be observed with the mutant when used at 10^7 CFU. This result indicates that the *iuc* mutant of M90T requires a higher inoculum to reach the threshold for maximal response observed with the wild-type strain. Figure 5A shows one typical experiment: intraluminal fluid accumulation was equivalent when M90T was inoculated at 10^7 CFU (loop 1) and its *iuc* mutant was used at 10^9 CFU (loop 4). Slight fluid accumulation was observed when the *iuc* mutant was inoculated at 10^7 CFU (loop 3), whereas the noninvasive plasmidless mutant BS 176 inoculated at 10^9 CFU (loop 2) caused no significant fluid accumulation.

Infected loops were then observed for gross alterations of the mucosa (Fig. 5B). In loop 1, infected by the wild-type M90T at 10^7 CFU, severe lesions could be observed, with disappearance of the normal folding of the mucosa; an intense purulent exudate was also observed. Loop 2 was a negative control which showed the normal aspect of the mucosal surface after inoculation with the noninvasive mutant BS176. Observation of loop 3 showed that after infection by the *iuc* mutant of M90T at 10^7 CFU, although fluid production was dramatically reduced, gross mucosal lesions could still be observed. These lesions were much less severe than those observed with the wild-type strain at the same inoculum; no major destruction or purulent exudate could be observed.

Figure 5C shows the histopathological alterations observed after infection with M90T and its *iuc* mutant. Only slight alterations could be observed in tissues of loops infected with the *iuc* mutant at 10^7 CFU (Fig. 5C1): the epithelial layer appeared intact with enlargement and shortening of the villus as a result of edematous swelling of the lamina propria. At 10^9 CFU (Fig. 5C2), the alterations generated by the *iuc* mutant were more severe: in addition to the previous lesions, the epithelium was infiltrated by polymorphonuclear and mononuclear leukocytes, which in many

TABLE 1. Results of the Sereny test with different inocula of strains M90T and M90T*iuc*::Tn*10*

Inoculum (CFU)	Result[a] with strain:							
	M90T after day:				M90T*iuc*::Tn*10* after day:			
	1	2	3	4[b]	1	2	3	4[b]
10^8	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
10^7	+ +	+ + +	+ + +	+ + +	0	+ +	+ + +	+ + +
10^6	0	0	+ +	+ + +	0	0	0	0
10^5	0	0	0	0	0	0	0	0

[a] Symbols: 0, no reaction; + +, keratoconjunctivitis; + + +, keratoconjunctivitis with important purulent exudate.
[b] Further observation revealed no delayed positive results.

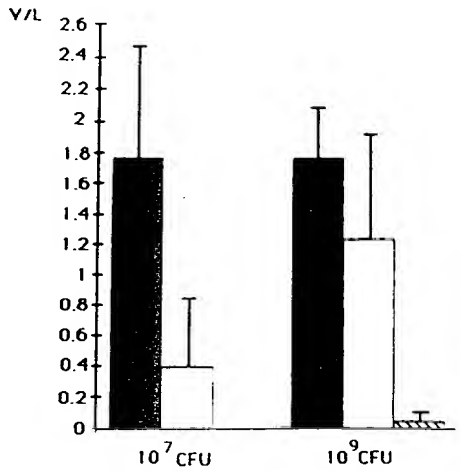

FIG. 4. Fluid production by infected loops. Production of fluid was expressed as the ratio of volume (in milliliters) to the length of the loop (in centimeters). Symbols: ▬, M90T; ▭, M90T*iuc*::Tn*10*; ▨, BS176. For each point the mean value and standard deviation of a minimum of four independent experiments are reported.

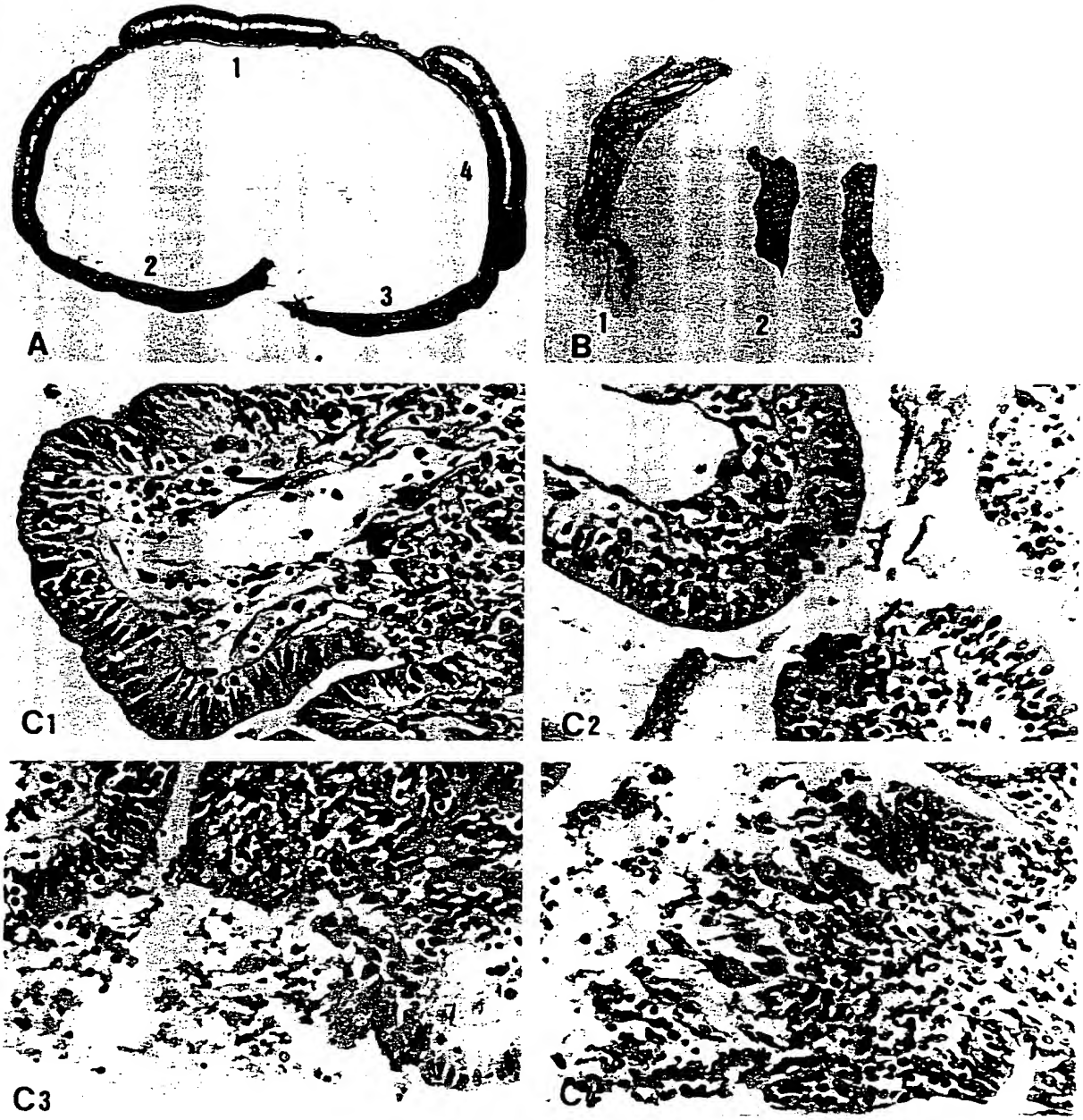

FIG. 5. (A and B) Macroscopic features of infected loops. Loop 1, M90T at 10^7 CFU; loop 2, BS176 at 10^9 CFU; loop 3, M90T*iuc*::Tn*10* at 10^7 CFU; loop 4, M90T*iuc*::Tn*10* at 10^9 CFU. (C) Histological aspect of infected loops. Panel C1 shows M90T*iuc*::Tn*10* at 10^7 CFU; panel 5C2 shows M90T*iuc*::Tn*10* at 10^9 CFU; panel 5C3 shows M90T at 10^7 CFU; panel 5C4 shows M90T at 10^9 CFU.

places generated abscesses and ulcerations. Histopathological alterations observed in loop tissues infected by M90T were similar and were independent of the inoculum (Fig. 5C3 and 5C4). The epithelial layer was disrupted by an intense infiltration of polymorphonuclear and mononuclear leukocytes with almost total destruction of the villus. Such an inoculum effect led us to evaluate whether the *iuc* mutation could lead to poor intraluminal survival or multiplication of the strain.

To assess the potential role of aerobactin production in intraluminal survival or growth, ligated rabbit ileal loops were infected with BS176 and BS176*iuc*::Tn*10* at two inocula (10^7 and 10^9 CFU). These strains could not invade the mucosa, and so we could explore the intraluminal compartment only. Intraluminal bacteria were then enumerated as described in Materials and Methods. At an initial inoculum of 10^7 CFU, BS176 and BS176*iuc*::Tn*10* reached a level of $(3 \pm 2.3) \times 10^8$ and $(2.38 \pm 1.2) \times 10^8$ CFU, respectively, after 24 h. At an initial inoculum of 10^9 CFU, BS176 and BS176*iuc*::Tn*10* reached a level of $(7.2 \pm 5.1) \times 10^8$ and $(4.4 \pm 4) \times 10^8$ CFU, respectively, after 24 h. These results represent the mean and standard deviation of six experi-

ments. There was no significant difference in intraluminal survival and multiplication between the two strains at the two inocula used.

DISCUSSION

Siderophore production is generally considered a virulence factor in bacteria. This has been clearly documented for the hydroxamate-type siderophore aerobactin in extracellular pathogens such as *E. coli* (28, 29) and *Klebsiella pneumoniae* (17). On the other hand, the contribution of siderophores to virulence during the intracellular stage of infection by facultative intracellular pathogens remains unclear. Recent work demonstrated that a mutation which blocked production of the phenolate-type siderophore enterochelin did not affect the virulence of *Salmonella typhimurium* in mice (2). In other facultative intracellular pathogens such as *Yersinia* spp., no siderophore has even been detected so far (19).

As the species *S. flexneri* produces aerobactin, it appeared important to assess the role of this siderophore in virulence and eventually determine the stage(s) of the overall infectious process at which it is acting. Shigellosis encompasses a complex series of events during which the invading microorganism goes from a stage of extracellular survival and growth in the lumen to a stage of intracellular growth within the epithelial layer and then to a final stage of growth within conjunctive tissues, which, for bacteria which are not phagocytosed, corresponds to extracellular growth.

In this study, a mutant of M90T, an *S. flexneri* strain which no longer produced aerobactin, was obtained through transposition of Tn*10*. Comparison of the hybridization patterns of a DNA probe which comprises the genes for aerobactin production with those of the chromosomal DNA of M90T and its *iuc*::Tn*10* mutant cleaved with various restriction enzymes confirmed that the transposon had inserted within the structural genes for hydroxamate biosynthesis. Like the parental strain, this mutant did not produce detectable amounts of enterochelin and, as a consequence, could not grow in iron-depleted medium.

The effect of this mutation was then tested both in vitro and in vivo. The in vitro assay, in which cultures of HeLa cell monolayers were infected by the parental strain or the *iuc* mutant, demonstrated that aerobactin does not influence growth within cells. This result was in agreement with those recently reported by Lawlor et al., who also showed that these bacteria can utilize hemin or hematin as a sole source of iron, thus allowing growth in the absence of siderophore synthesis (14). In addition, the ability of the *iuc*::Tn*10* mutant to kill HeLa cells as efficiently as its parental strain indicated that the early killing process was not mediated by iron depletion of the intracellular compartment.

Of the in vivo assays, the Sereny test demonstrated that introduction of the *iuc*::Tn*10* mutation had an inoculum-dependent effect on the outcome of keratoconjunctival infection. The production of aerobactin seemed to provide the strain with a selective advantage. However, such a test did not identify the stage of the infectious process at which the selective advantage provided by aerobactin production may operate. Infection of ligated segments of rabbit ileal loops, which represents a more definitive assay, allowed us to assess the effect of the mutation with more accuracy. After 18 h, equivalent numbers of bacteria were found when the loop was infected with the noninvasive derivative of M90T, BS176, and its *iuc*::Tn*10* mutant. Such results suggest that aerobactin production does not provide strains with a selective advantage at the stage of intraluminal survival and growth. Similar experiments were conducted with invasive M90T and its *iuc*::Tn*10* mutant to monitor the loops for fluid production, gross alterations of the mucosal surface, and intensity of histopathological lesions. An inoculum-dependent effect was again observed with minor alterations for the mutant at an inoculum of 10^7 CFU, whereas the parental strain displayed a full pattern of virulence at a similar inoculum. On the other hand, lesions appeared qualitatively similar although quantitatively smaller when the mutant was used at an inoculum of 10^9 CFU per loop.

If one considers that *S. flexneri* behavior in HeLa cells is a reflection of the actual situation within intestinal cells, such experiments would indicate that the selective advantage provided by aerobactin production operates at the stage of multiplication within tissues when bacteria lie within the extracellular compartment of the intestinal villus.

Such a mutant may be worth considering as a component of a vaccine. It could not be expected by itself to provide sufficient attenuation of virulence, but it would certainly be considered an additional security.

ACKNOWLEDGMENTS

We thank N. Nassif and A. Fondimare for invaluable help in histopathological studies, J. Arondel for outstanding expertise in rabbit surgery, and H. d'Hauteville for excellent technical help.

This work was supported by a grant from the Diarrheal Diseases Control Programme of the World Health Organization and by constant financial support from Pasteur Vaccins.

LITERATURE CITED

1. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
2. **Benjamin, W. H., Jr., C. L. Turnbough, Jr., B. S. Posey, and D. E. Briles.** 1985. The ability of *Salmonella typhimurium* to produce the siderophore enterobactin is not a virulence factor in mouse typhoid. Infect. Immun. **50**:392–397.
3. **Bindereif, A., and J. B. Neilands.** 1983. Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. J. Bacteriol. **153**:1111–1113.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
6. **Carbonetti, N. H., and P. H. Williams.** 1985. Detection of synthesis of the hydroxamate siderophore aerobactin by pathogenic isolates of *Escherichia coli*, p. 419–424. *In* M. Sussman (ed.), The virulence of *Escherichia coli*, Reviews and methods. Academic Press, Inc., Orlando, Fla.
7. **Clerc, P. L., A. Ryter, J. Mounier, and P. J. Sansonetti.** 1987. Plasmid-mediated early killing of eucaryotic cells by *Shigella flexneri* as studied by infection of J774 macrophages. Infect. Immun. **55**:521–527.
8. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
9. **Griffiths, E., P. Stervenson, T. L. Hale, and S. B. Formal.** 1985. Synthesis of aerobactin and a76,000-dalton iron-regulated outer membrane protein by *Escherichia coli* K-12–*Shigella flexneri* hybrids and by enteroinvasive strains of *Escherichia coli*. Infect. Immun. **49**:67–71.
10. **Hibbs, J. B., Jr., R. R. Taintor, and Z. Vavrin.** 1984. Iron depletion: possible cause of tumor cell cytotoxicity induced by activated macrophages. Biochem. Biophys. Res. Commun. **123**:716–723.

11. **Jorgensen, R. A., D. E. Berg, B. Allet, and W. S. Reznikoff.** 1979. Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. J. Bacteriol. 137: 681–685.
12. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365–1373.
13. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering in vivo using translocatable drug-resistance elements: new methods in bacterial genetics. J. Mol. Biol. 116:125–159.
14. **Lawlor, K. M., P. A. Daskaleros, R. E. Robinson, and S. M. Payne.** 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. 55:594–599.
15. **Lawlor, K. M., and S. M. Payne.** 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. 160:266–272.
16. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology 1:190–206.
17. **Nassif, X., and P. J. Sansonetti.** 1986. Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. Infect. Immun. 54:603–608.
18. **Payne, S. M., D. W. Niesel, S. S. Peixotto, and K. M. Lawlor.** 1983. Expression of hydroxamate and phenolate siderophores by *Shigella flexneri*. J. Bacteriol. 155:949–955.
19. **Perry, R. D., and R. R. Brubaker.** 1979. Accumulation of iron by yersiniae. J. Bacteriol. 137:1290–1298.
20. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.
21. **Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten.** 1963. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 604:5.
22. **Roussel, A. F., and Y. A. Chabbert.** 1978. Taxonomy and epidemiology of Gram-negative bacterial plasmids studied by DNA-DNA filter hybridization in formamide. J. Gen. Microbiol. 104:269–276.
23. **Sansonetti, P. J., T. L. Hale, G. I. Dammin, C. Kapper, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenesis of *Escherichia coli* K-12 after transfer of plasmids and chromosomal genes from *Shigella flexneri*. Infect. Immun. 39: 1392–1402.
24. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. 35:852–860.
25. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* with HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. 51:461–469.
26. **Sereny, B.** 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. 2:293–296.
27. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
28. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. Infect. Immun. 26:925–932.
29. **Williams, P. H., and P. J. Warner.** 1980. ColV plasmid-mediated colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. Infect. Immun. 29:411–416.